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# DNA 3'-Phosphatase Activity Is Critical for Rapid Global Rates of Single-Strand Break Repair following Oxidative Stress<sup>†</sup>

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**Oxidative stress is a major source of chromosome single-strand breaks (SSBs), and the repair of these lesions is retarded in neurodegenerative disease. The rate of the repair of oxidative SSBs is accelerated by XRCC1, a scaffold protein that is essential for embryonic viability and that interacts with multiple DNA repair proteins. However, the relative importance of the interactions mediated by XRCC1 during oxidative stress in vivo is unknown. We show that mutations that disrupt the XRCC1 interaction with DNA polymerase  $\beta$  or DNA ligase III fail to slow SSB repair in proliferating CHO cells following oxidative stress. In contrast, mutation of the domain that interacts with polynucleotide kinase/phosphatase (PNK) and Aprataxin retards repair, and truncated XRCC1 encoding this domain fully supports this process. Importantly, the impact of mutating the protein domain in XRCC1 that binds these end-processing factors is circumvented by the overexpression of wild-type PNK but not by the overexpression of PNK harboring a mutated DNA 3'-phosphatase domain. These data suggest that DNA 3'-phosphatase activity is critical for rapid rates of chromosomal SSB repair following oxidative stress, and that the XRCC1-PNK interaction ensures that this activity is not rate limiting in vivo.**

Oxidative stress can have a major influence on genome integrity and cell survival and is an etiological factor in a number of neurological human diseases. Of these, several are associated with defects in the repair of DNA damage, including xeroderma pigmentosum (XP), ataxia telangiectasia (A-T), ataxia oculomotor apraxia 1 (AOA1), and spinocerebellar ataxia with axonal neuropathy 1 (SCAN1) (1, 28, 34, 37). The neuropathology evident in XP most likely reflects an inability to repair one or more single-strand oxidative adducts by nucleotide excision repair. In contrast, A-T is associated with cellular defects in the repair of DNA double-strand breaks (DSBs) and AOA1 and SCAN1 with defects in the repair of DNA single-strand breaks (SSBs). SSBs are the commonest DNA lesions arising in cells, and if they are not rapidly repaired they can inhibit transcription and/or generate replication-associated DSBs (3, 26, 59, 60). The repair of oxidative SSBs involves DNA damage detection by PARP-1 followed by recruitment of the enzymes required for subsequent steps of the repair process, which include DNA end processing, DNA gap filling, and DNA ligation (9, 19). Many of the enzymes implicated in these steps interact physically with XRCC1, including DNA polynucleotide kinase (PNK) (54), Aprataxin (APTX) (13, 14, 18, 31, 44), DNA polymerase  $\beta$  (Pol  $\beta$ ) (10, 27), and DNA ligase III $\alpha$  (Lig3 $\alpha$ ) (11, 12). This has prompted the hypothesis that XRCC1 is a scaffold protein that recruits, stabilizes, and/or stimulates SSB repair (SSBR) enzymes at chromosomal SSBs, thereby accelerating the overall process (8, 9). While in vitro analyses generally are consistent with this idea, including the observation that XRCC1 mutation (50, 58),

deletion (49), or depletion (6) retards the rate of chromosomal SSBR by approximately fivefold following DNA oxidation or DNA base damage, the relative importance of the protein-protein interactions mediated by XRCC1 for SSBR is unclear.

Here, we have addressed the importance of the protein-protein interactions mediated by XRCC1 during the repair of oxidative SSBs. To do this, we have employed isogenic XRCC1 mutant CHO cells expressing recombinant derivatives of XRCC1 in which specific protein-protein interaction domains are mutated. We find that whereas the interactions between XRCC1 and either Pol  $\beta$  or Lig3 $\alpha$  are dispensable for rapid rates of chromosomal SSBR in asynchronous populations of CHO cells following oxidative stress, SSBR rates are markedly slowed in cells expressing XRCC1 that cannot interact with PNK. Importantly, we show that the overexpression of wild-type recombinant PNK, but not 3'-phosphatase-dead PNK, can override the requirement for PNK interaction with XRCC1 for rapid rates of SSBR following oxidative stress. These data indicate that DNA 3'-phosphatase activity is critical for rapid rates of chromosomal SSBR following oxidative stress, and that the XRCC1 interaction with PNK prevents this activity from becoming rate limiting.

## MATERIALS AND METHODS

**DNA constructs.** pCD2E-XH<sup>F67A</sup>, pCD2E-XH<sup>S485A,T488A</sup>, and pCD2E-XH<sup>S518A,T519A,T523A</sup> were created by the site-directed mutagenesis of the XRCC1 open reading frame (ORF) in pCD2E-XH (12) using a QuikChange mutagenesis kit (Stratagene) and the appropriate primers. pCD2E and pCD2E-XH<sup>CKM</sup> have been described previously (30, 46). To create pCD2E-XH<sup>S408A,S409A,S410A</sup>, a KpnI fragment of wild-type XRCC1 cDNA from pCD2E-XH was replaced with the corresponding fragment in pET16B-XH<sup>S408A,S409A,S410A</sup> (Richard Taylor, unpublished data). To create pAS-XRCC1<sup>F67A</sup>, a CeliI-BlnI fragment of XRCC1 from pAS-XH was replaced with the corresponding fragment from pCD2E-XH<sup>F67A</sup>. To create pCD2E-PNK<sup>D171N</sup>, the PNK ORF in pCD2E-PNK (30) was mutated by site-directed mutagenesis as described above. pCD2E-HX<sup>161-533</sup> encoding His-XRCC1<sup>161-533</sup> was created by PCR amplification, insertion into pCR2.1-TOPO (Invitrogen), and subcloning into the EcoRI sites of pCD2E.

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**Cell lines.** The XRCC1 mutant CHO cell line EM9 and derivatives were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, 2 mM glutamine, and 100 µg/ml streptomycin. Expression constructs were introduced into EM9 cells by calcium phosphate coprecipitation (EM9-XH<sup>F67A</sup>) or by Genejuice (Novagen) transfection (EM9-XH<sup>CKM</sup>, EM9-XH<sup>S408A,S409A,S410A</sup>, EM9-XH<sup>S485A,T488A</sup>, and EM9-XH<sup>S518A,T519A,T523A</sup>). Stable cell lines were prepared by the selection of transfected cells with 1.5 mg/ml G418 (Gibco-Invitrogen) for 7 to 10 days.

**Antibodies and immunoblotting.** Cells were lysed in sodium dodecyl sulfate (SDS) loading buffer at 90°C, and whole-cell extracts from  $5 \times 10^4$  to  $2.5 \times 10^5$  cells were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose and immunoblotted with anti-XRCC1 monoclonal (clone 33.2.5), anti-XRCC1 polyclonal (SK3188), anti-PNK (SK3195), anti-Lig3α polyclonal (TL25), anti-Polβ polyclonal (a kind gift from Grigory Dianov), or anti-β-actin monoclonal antibody (Sigma).

**Yeast two-hybrid analyses.** *Saccharomyces cerevisiae* Y190 cells were transformed with the indicated pAS and pACT constructs, and transformants were selected on yeast minimal medium plates (glucose plus yeast nitrogen base without amino acids) supplemented with adenine and histidine. Pooled populations of transformants were streaked onto minimal medium supplemented with either adenine and histidine for β-galactosidase colony-lift filter assays or adenine and 25 mM 3-amino-1,2,4-triazole for the analysis of histidine prototrophy.

**Affinity purification of histidine-tagged XRCC1 protein complexes from CHO cells.** For the analysis of the XRCC1-Pol β interaction, whole-cell extract was prepared from a confluent flask (T75) or plate (15 cm) of EM9-XH, EM9-XH<sup>F67A</sup>, or EM9-V cells by resuspension in lysis buffer (25 mM HEPES, pH 8.0, 325 mM sodium chloride, 0.5% Triton X-100, 10% glycerol, 1 mM dithiothreitol [DTT], 25 mM imidazole, 1/100 dilution of mammalian protease inhibitor cocktail [Sigma]) at a density of  $6 \times 10^6$  cells/ml and incubated on ice for 15 min. High-molecular-weight DNA was sheared by passage through a narrow-gauge needle, and extracts were clarified by centrifugation ( $8,832 \times g$ ) for 10 min at 2°C. For the analysis of the XRCC1-PNK interaction, EM9 cells were transiently transfected with 8 µg/dish each of pCD2E-PNK and either pCD2E-XH, pCD2E-XH<sup>CKM</sup>, pCD2E-XH<sup>S408A,S409A,S410A</sup>, pCD2E-XH<sup>S485A,T488A</sup>, pCD2E-XH<sup>S518A,T519A,T523A</sup>, or empty pCD2E using Genejuice (Novagen). Twenty-four hours later, transiently transfected cell populations were selected in medium containing 1.5 mg/ml G418 for 4 days. Cells ( $5 \times 10^6$ ) were harvested, washed, and resuspended in 0.3 ml lysis buffer (25 mM HEPES, pH 8.0, 325 mM sodium chloride, 10% glycerol, 1 mM DTT, 25 mM imidazole, 1/100 dilution of mammalian protease inhibitor cocktail [Sigma]) and sonicated (five 30-s pulses at high power using a Biorupter sonicator). XRCC1-His complexes then were purified from the clarified extract by metal-chelate affinity chromatography. Briefly, cell extract (0.3 to 1 ml) was incubated with 0.25 to 0.5 ml nickel-nitrilotriacetic acid-agarose (Qiagen) for 20 min on ice with frequent mixing. The resulting suspension was added to a disposable 5-ml chromatography column (Polyprep; Bio-Rad), and the flow rate was adjusted to ~0.5 ml/min. The column was washed (twice with 5 ml) with lysis buffer and then 5 ml of elution buffer (lysis buffer containing 250 mM imidazole), and 0.5-ml fractions were collected.

**Clonogenic survival assays.** For survival assays, five hundred cells were plated in 10-cm dishes in duplicate and incubated for 4 h at 37°C. Cells were rinsed with phosphate-buffered saline (PBS) (twice) and either mock treated or treated with H<sub>2</sub>O<sub>2</sub> (diluted in PBS at the appropriate concentration immediately prior to use) for 15 min at room temperature or with MMS (diluted in complete medium at the appropriate concentration immediately prior to use) for 15 min at 37°C. After treatment, cells were washed twice with PBS and incubated for 10 to 14 days in drug-free medium at 37°C to allow the formation of macroscopic colonies. Colonies were fixed in ethanol (95%) and stained with 1% methylene blue–70% ethanol. Colonies of greater than 50 cells were counted, and the survival (as a percentage) was calculated using the equation  $100 \times [\text{mean colony number (treated plate)}/\text{mean colony number (untreated plate)}]$ .

**Alkaline single-cell agarose-gel electrophoresis (alkaline comet assay).** To examine EM9-XH<sup>F67A</sup> and its associated controls by alkaline comet assay,  $2 \times 10^5$  cells were plated in 3.5-cm culture dishes in complete medium and incubated for 16 to 18 h at 37°C prior to mock treatment or treatment with 0.15 to 0.2 mM H<sub>2</sub>O<sub>2</sub> in PBS for 10 to 20 min on ice. For other cell lines, subconfluent monolayers were trypsinized and diluted to  $4 \times 10^5$  cells/ml immediately prior to treatment with H<sub>2</sub>O<sub>2</sub> as described above. For exposure with gamma rays, cells were treated in suspension ( $4 \times 10^5$  cells/ml) in complete medium. After treatment with MMS, H<sub>2</sub>O<sub>2</sub>, or gamma irradiation (20 Gy), cells were washed once in ice-cold PBS and incubated in fresh drug-free medium for the desired repair period. Adherent cells were harvested in ice-cold PBS by scraping (1 ml/3.5-cm plate), and 0.2 ml of the cell suspension was analyzed by alkaline comet assay as

previously described (7). For cells treated in suspension, 1-ml aliquots ( $4 \times 10^5$  cells) were analyzed as described above. For transient-transfection experiments, EM9 cells were transiently cotransfected in 10-cm dishes with 8 µg/dish of either pCD2E-XH, pCD2E-XH<sup>S518A,T519A,T523A</sup>, or empty pCD2E and additionally with 24 µg/dish of either pCD2E-PNK, pCD2E-PNK<sup>D171N</sup>, or empty pCD2E using Genejuice (Novagen). Twenty-four hours later cells were selected in medium containing 1.5 mg/ml G418 for 4 days and then H<sub>2</sub>O<sub>2</sub> treated or not treated as described above.

## RESULTS

XRCC1 interacts with polypeptide components of each of the three core stages of DNA processing during SSBR (Fig. 1A). However, the relative importance of these interactions for the repair of oxidative SSBs is unknown. To examine the requirement for XRCC1 interaction with Lig3α during DNA ligation, we employed XRCC1 mutant EM9 CHO cells stably expressing either wild-type recombinant human XRCC1-His (EM9-XH cells), XRCC1-His<sup>8529-633</sup> protein lacking the C-terminal BRCT domain that binds Lig3α (EM9-XH<sup>8529-633</sup> cells), or an empty expression vector (EM9-V cells) (Fig. 1B). We have shown previously that the deletion of the C-terminal BRCT domain ablates binding to Lig3α (36). Consistent with this, Lig3 levels were no higher in EM9-XH<sup>8529-633</sup> cells than in EM9-V cells, consistent with the requirement for interaction with XRCC1 for Lig3α stability (Fig. 1B) (11, 12). Notably, while EM9-V cells harboring empty vector were hypersensitive to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress, the level of sensitivity of EM9-XH<sup>8529-633</sup> cells to H<sub>2</sub>O<sub>2</sub> was similar to that of EM9-XH (Fig. 1C). To measure chromosomal SSBR rates, we employed alkaline comet assays. Although this assay can detect both SSBs and DSBs, the vast majority (>99%) of breaks induced by H<sub>2</sub>O<sub>2</sub> are SSBs (4). Surprisingly, while EM9-V cells harboring empty vector exhibited reduced rates of global SSBR following H<sub>2</sub>O<sub>2</sub>-induced oxidative stress, SSBR rates in EM9-XH<sup>8529-633</sup> cells were similar to those of EM9-XH (Fig. 1D). We conclude that the interaction between XRCC1 and Lig3α is dispensable for the repair of oxidative SSBs in proliferating CHO cells.

To examine the requirement for XRCC1 interaction with DNA Pol β, we employed EM9 cells expressing XRCC1 protein harboring the point mutation F67A (Fig. 2A). This mutation is located within the N-terminal domain (NTD) that binds Pol β and reduces the interaction of XRCC1 with the DNA polymerase in vitro (33). Consistent with this, the F67A mutation reduced Pol β binding by XRCC1 more than 25-fold in vivo, whereas the interaction with aprataxin (APTX), which is mediated by the C-terminal half of XRCC1, was only weakly (<2-fold) affected (data not shown; also see Fig. S1A in the supplemental material). In addition, whereas Pol β copurified with XRCC1-His from EM9-XH cell extract, it failed to do so measurably from cell extract from EM9 cells expressing XRCC1-His<sup>F67A</sup> (denoted EM9-XH<sup>F67A</sup> cells) (see Fig. S1B in the supplemental material). XRCC1-His<sup>F67A</sup> largely corrected the hypersensitivity of XRCC1-deficient EM9 cells to H<sub>2</sub>O<sub>2</sub>, suggesting that the XRCC1-Pol β interaction is dispensable for cell survival following oxidative DNA damage (Fig. 2B). XRCC1-His<sup>F67A</sup> similarly restored cellular resistance to H<sub>2</sub>O<sub>2</sub> in EM-C11 cells, a second XRCC1 mutant CHO cell line (see Fig. S2 in the supplemental material). XRCC1-His<sup>F67A</sup> also complemented the defect in chromosomal SSBR rates in

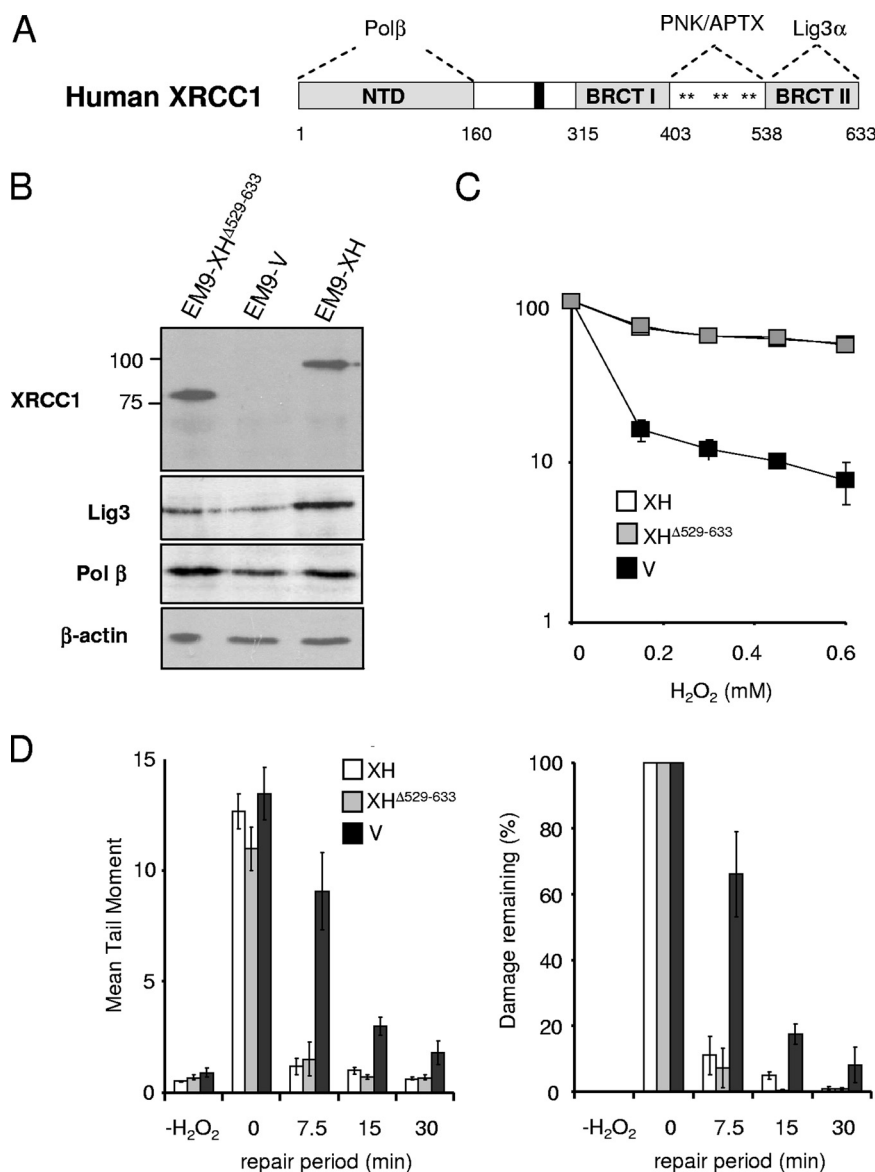


FIG. 1. XRCC1-DNA ligase III $\alpha$  interaction is dispensable for rapid SSBR rates following oxidative stress in proliferating CHO cells. (A) Schematic depicting human XRCC1 and the protein domains that mediate interaction with Pol  $\beta$ , PNK, APTX, and Lig3 $\alpha$ . Asterisks denote the three clusters of consensus CK2 phosphorylation sites. (B) Cell extracts from the indicated cell lines were fractionated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-XRCC1 monoclonal, anti-Lig3 polyclonal, anti-Pol  $\beta$  polyclonal, or anti- $\beta$ -actin monoclonal antibody. (C) Clonogenic sensitivity of the indicated EM9 CHO cell lines to H<sub>2</sub>O<sub>2</sub>. Cells were treated for 15 min at room temperature with the indicated concentrations of H<sub>2</sub>O<sub>2</sub> in PBS, and surviving colonies were stained and counted after 10 to 14 days. Data are the means ( $\pm$  standard errors of the means) of at least three independent experiments. (D) DNA strand break repair rates following H<sub>2</sub>O<sub>2</sub> treatment as measured by alkaline comet assays. The indicated EM9 CHO cell lines were treated with 150  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 20 min on ice in PBS and then incubated for the indicated repair periods in drug-free media. DNA strand breaks were quantified by alkaline comet assays from three or more independent experiments. (Left) Mean tail moments were quantified from  $\sim$ 100 cells/sample/experiment and are the averages ( $\pm$  standard errors of the means) of at least three independent experiments. (Right) The data in the left panel were plotted as the mean fractions of DNA strand breaks remaining at the indicated time points.

XRCC1 mutant cells, because H<sub>2</sub>O<sub>2</sub>-induced DNA breaks declined at normal rates in EM9-XH<sup>F67A</sup> cells compared to those of EM9-V cells (Fig. 2C). This result was not limited to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress, because similar results were observed following ionizing radiation (Fig. 2D). Notably, in contrast to oxidative stress, XRCC1-His<sup>F67A</sup> failed to fully correct cellular hypersensitivity and reduced repair rates in EM9 cells following methyl methanesulfonate (MMS) treatment, consistently

with the established importance of the XRCC1-Pol  $\beta$  interaction during DNA base excision repair (BER) (15, 38, 56) (Fig. 2E and F). We conclude that while the XRCC1-Pol  $\beta$  interaction is required for efficient BER, it is dispensable for cell survival and rapid rates of chromosomal SSBR in proliferating cells following oxidative stress.

To confirm that the requirement for XRCC1 for rapid rates of SSBR in asynchronous CHO cells did not require interac-



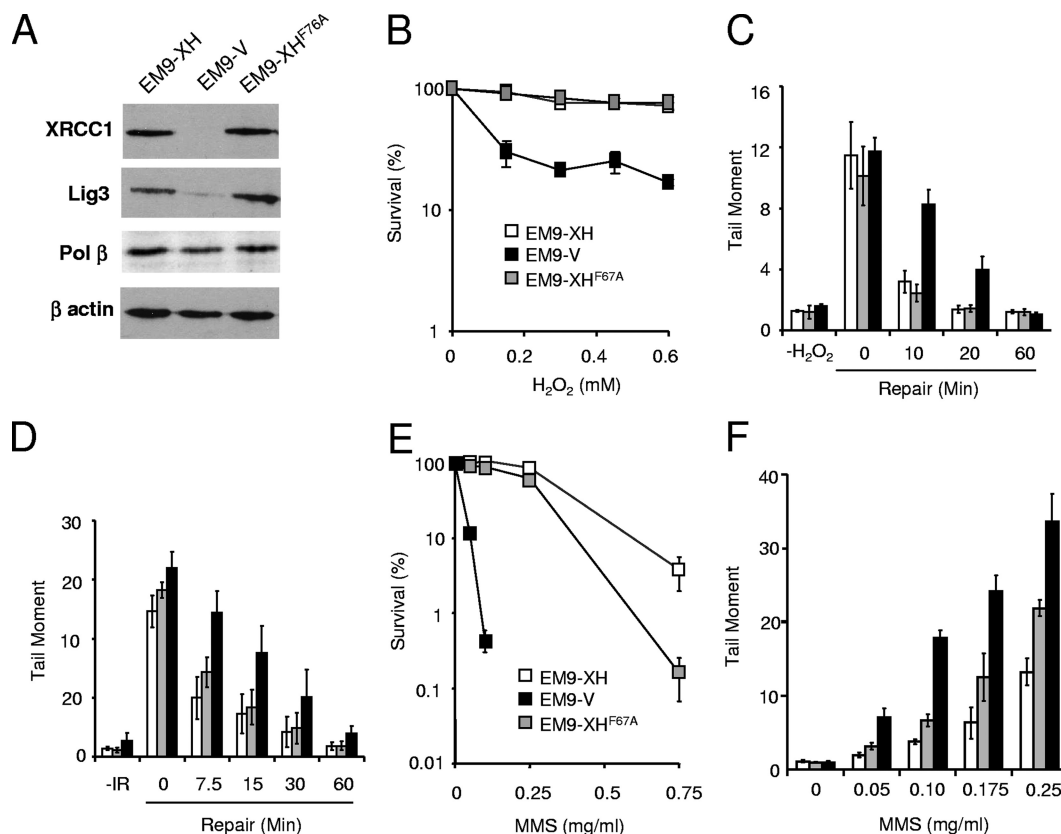


FIG. 2. XRCC1-Pol  $\beta$  interaction is dispensable for rapid SSBR rates following oxidative stress in proliferating CHO cells. (A) Cell extracts from the indicated cell lines were fractionated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-XRCC1 monoclonal, anti-Lig3 polyclonal, anti-Pol  $\beta$  polyclonal, or anti- $\beta$ -actin monoclonal antibody. (B) Clonogenic sensitivity of the indicated cell lines to H<sub>2</sub>O<sub>2</sub>. Cells were treated for 15 min at room temperature with the indicated concentrations of H<sub>2</sub>O<sub>2</sub> in PBS, and surviving colonies were stained and counted after 10 to 14 days. Data are the means ( $\pm$  standard errors of the means) of at least three independent experiments. (C) DNA strand break repair rates following H<sub>2</sub>O<sub>2</sub> treatment. The indicated CHO cell lines (symbols as in panel B) were treated with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 10 min on ice and then incubated for the indicated repair periods in drug-free media. DNA strand breaks were quantified by alkaline comet assays as described in the legend to Fig. 1. Mean tail moments were quantified from  $\sim$ 100 cells/sample/experiment and are the averages ( $\pm$  standard errors of the means) of at least three independent experiments. (D) DNA strand break repair rates following gamma radiation. The indicated CHO cell lines (symbols as in panel B) were treated with irradiation (IR) (20 Gy) and then incubated for the indicated repair periods in drug-free media. DNA strand breaks were quantified as described for panel C. (E) Clonogenic sensitivity of the indicated cell lines to MMS. Cells were treated for 15 min at 37°C with the indicated concentrations of MMS, and surviving colonies stained and counted after 10 to 14 days. Data are the means ( $\pm$  standard errors of the means) of at least three independent experiments. (F) DNA strand break repair rates following MMS treatment. The indicated CHO cell lines (symbols as in panel B) were treated with the indicated concentrations of MMS for 15 min at 37°C, and the accumulated levels of DNA strand breaks were quantified as described for panel C.

tion with either Pol  $\beta$  or Lig3 $\alpha$ , we employed a truncated derivative of human XRCC1 (His-XRCC1<sup>161-533</sup>) lacking both the amino-terminal (NTD) and C-terminal domains that bind Pol  $\beta$  and Lig3 $\alpha$ , respectively (Fig. 3A). This protein mimics the native XRCC1 homolog that is present in the plant *Arabidopsis thaliana*, and it was expressed in EM9 cells at levels similar to those of wild-type human His-XRCC1 (Fig. 3B). Notably, proliferating EM9-HX<sup>161-533</sup> cells expressing the truncated protein exhibited both normal sensitivity (Fig. 3C) and normal rates of SSBR following H<sub>2</sub>O<sub>2</sub> treatment (Fig. 3D and E). These data identify the region spanning amino acids 161 to 533 as sufficient for the ability of XRCC1 to maintain cell survival and rapid global rates of SSBR in proliferating CHO cells.

Residues 161 to 533 encompass the most evolutionarily conserved region of XRCC1 and include the inter-BRCT domain linker region of  $\sim$ 100 amino acids (Fig. 3A). This region,

which is phosphorylated multiple times at CK2 consensus sites that are grouped into three clusters at residues 408/409/410, 485/488, and 518/519/523, mediates phosphorylation-specific interactions with the DNA end-processing factors PNK (30) and APTX (13, 31) and also with a recently identified protein of unknown function, denoted APLF (2, 22, 24). Previously, we mutated all eight of the predicted primary CK2 phosphorylation sites in XRCC1 and showed that the mutant protein (denoted XRCC1<sup>CKM</sup>) was unable to support normal rates of SSBR following oxidative stress (30). However, whether the importance of this region for SSBR reflects the interaction with PNK or with APTX and/or APLF is unknown. To address this issue, we mapped the site of PNK interaction in detail by mutating each of the three clusters of CK2 phosphorylation sites separately and expressing the respective proteins, denoted XRCC1-His<sup>S408A/S409A/S410A</sup>, XRCC1-His<sup>S485A/T488A</sup>, and XRCC1-His<sup>S518A/T519A/T523A</sup>, in EM9 cells (Fig. 4A). PNK

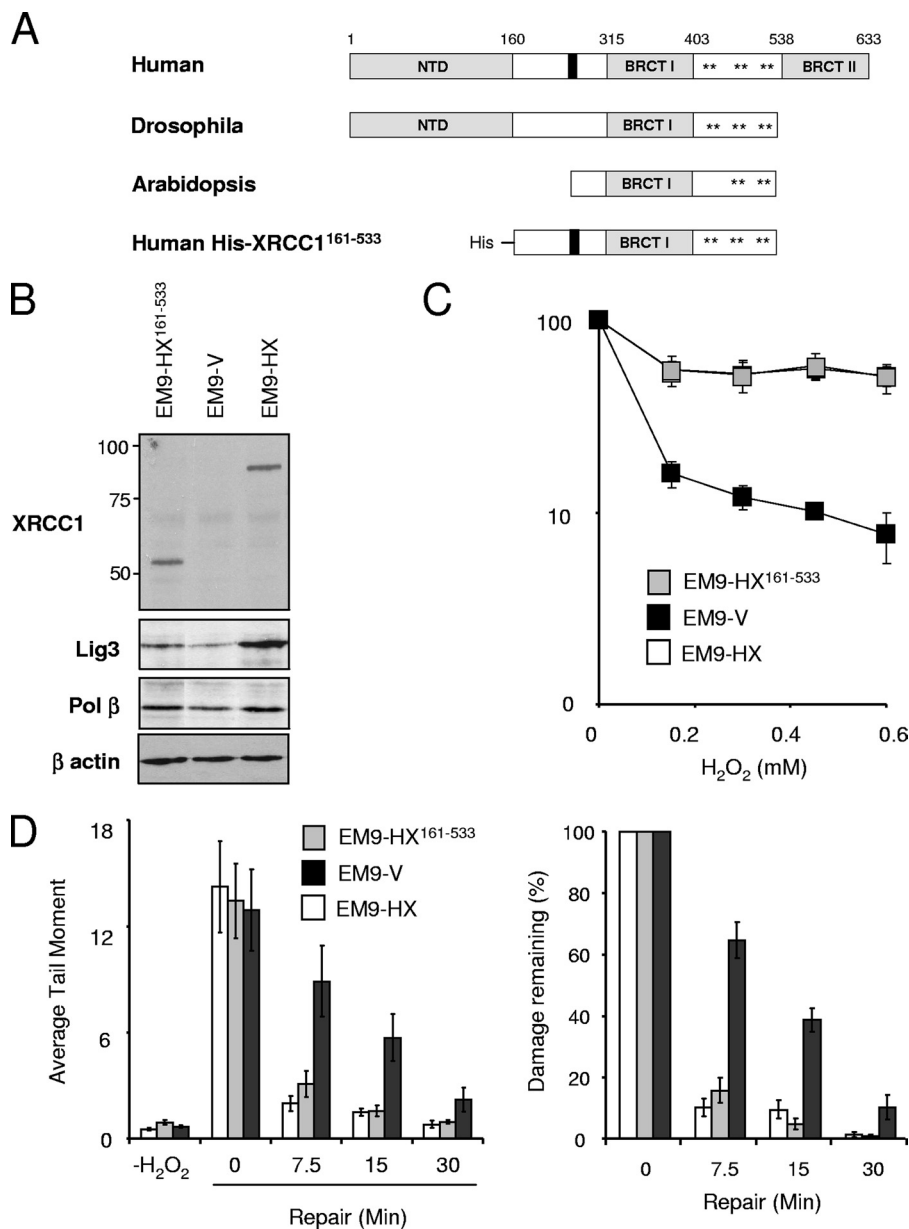


FIG. 3. His-XRCC1<sup>161-533</sup> restores cellular resistance and rapid SSBR rates in XRCC1 mutant CHO cells following oxidative stress. (A) Schematic depicting the evolutionarily conserved domains in XRCC1. XRCC1 homologues in *Homo sapiens*, *Drosophila melanogaster*, and *Arabidopsis thaliana* are shown, along with the truncated derivative of human XRCC1 employed here (denoted His-XRCC1<sup>161-533</sup>). Asterisks denote clusters of CK2 consensus phosphorylation sites. (B) XRCC1 protein levels in XRCC1 mutant CHO cells stably transfected with empty expression vector (EM9-V) or with expression constructs encoding either wild-type His-XRCC1 (EM9-HX) or mutant His-XRCC1 lacking the NTD and C-terminal BRCT II domain (EM9-HX<sup>161-533</sup>). Cell extracts from the indicated cell lines were fractionated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-XRCC1 monoclonal, anti-Lig3 polyclonal, anti-Pol β polyclonal, or anti-β-actin monoclonal antibody. (C) Clonogenic sensitivity of the indicated cell lines to H<sub>2</sub>O<sub>2</sub>. Cells were treated for 15 min at room temperature with the indicated concentrations of H<sub>2</sub>O<sub>2</sub> in PBS, and surviving colonies were stained and counted after 10 to 14 days. Data are the means (± standard errors of the means) of at least three independent experiments. (D) DNA strand break repair rates following H<sub>2</sub>O<sub>2</sub> treatment as measured by alkaline comet assays. The indicated CHO cell lines were treated with 150 μM H<sub>2</sub>O<sub>2</sub> for 20 min on ice and then incubated for the indicated repair periods in drug-free media. DNA strand breaks were quantified by alkaline comet assays as described in the legend to Fig. 1. (Left) Mean tail moments were quantified from ~100 cells/sample/experiment and are the averages (± standard errors of the means) of at least three independent experiments. (Right) The data in the left panel were plotted as the mean fractions of DNA strand breaks remaining at the indicated time points.

copurified with wild-type XRCC1-His, XRCC1-His<sup>S408A/S409A/S410A</sup>, and XRCC1-His<sup>S485A/T488A</sup> during affinity chromatography but failed to copurify with either XRCC1-His<sup>CKM</sup> or XRCC1-His<sup>S518A/T519A/T523A</sup> (Fig. 4B). Most of the recombinant

XRCC1 recovered in these experiments was truncated by ~10 kDa due to the proteolytic removal of the labile amino-terminal domain. This did not affect interaction with PNK, however, which occurs toward the C terminus of XRCC1. We conclude

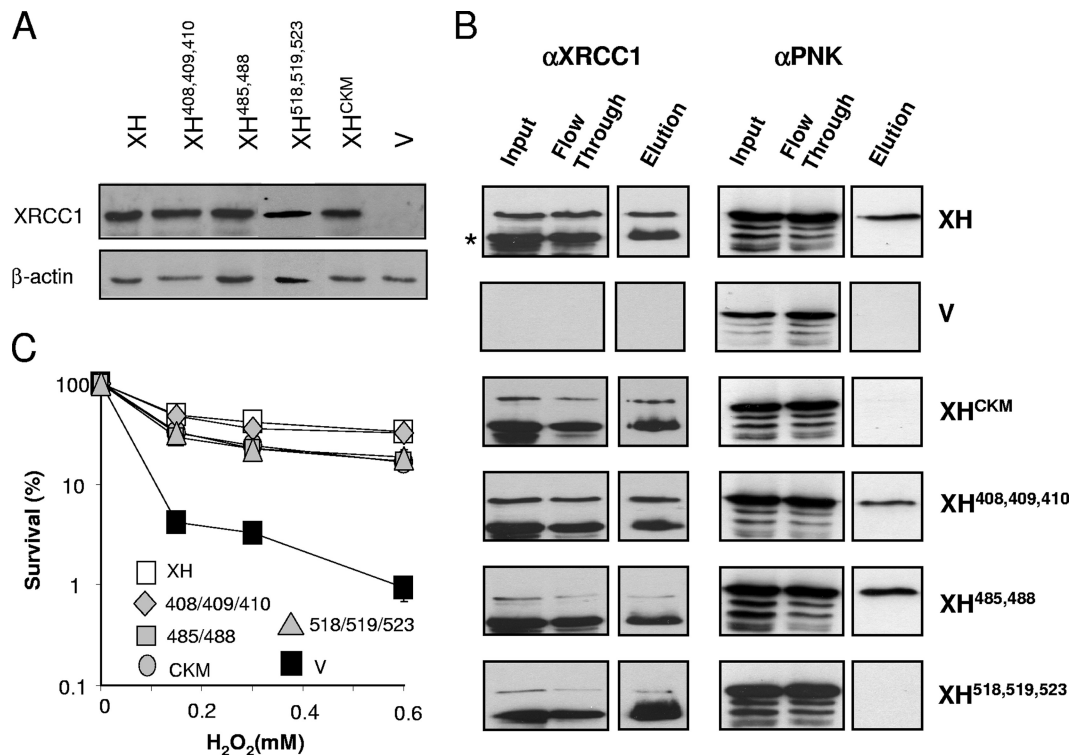


FIG. 4. Mutation of the cluster of CK2 phosphorylation sites at S518/T519/T523 greatly reduces or ablates interaction with PNK. (A) Total cell extracts from  $5 \times 10^4$  of cells expressing the indicated recombinant XRCC3 protein or harboring empty vector (v) were fractionated by SDS-PAGE, transferred to nitrocellulose, and immunostained with anti-XRCC1 polyclonal antibody or anti-β-actin monoclonal antibody. (B) XRCC1-His protein complexes were recovered from whole-cell extract from  $5 \times 10^6$  of the indicated cells by affinity chromatography as described in Materials and Methods. Aliquots of the column input (8% of column input), flowthrough, and 250 mM imidazole eluate (13% of total eluate) were fractionated by SDS-PAGE and immunoblotted for the presence of anti-XRCC1 (αXRCC1) or anti-PNK (αPNK) polyclonal antibody. (C) Clonogenic sensitivity of the indicated cell lines to H<sub>2</sub>O<sub>2</sub>. Cells were treated for 15 min at room temperature with the indicated concentrations of H<sub>2</sub>O<sub>2</sub> in PBS, and surviving colonies were stained and counted after 10 to 14 days. Data are the means of at least three independent experiments ( $\pm$  standard errors of the means).

from these experiments that the interaction between XRCC1 and PNK requires only the cluster of CK2 phosphorylation sites at S518/T519/T523. Notably, this is the same cluster of CK2 phosphorylation sites that interacts with APTX (31).

None of the CK2 phosphorylation site mutations greatly affected the ability of XRCC1 to correct the hypersensitivity of EM9 cells to H<sub>2</sub>O<sub>2</sub> (Fig. 4C). In contrast, global rates of SSBR were markedly slower in EM9-XH<sup>CKM</sup> and EM9-XH<sup>S518A/T519A/T523A</sup> cells than in EM9-XH, EM9-XH<sup>S408A/S409A/S410A</sup>, or EM9-XH<sup>S485A/T488A</sup> cells (Fig. 5A). This suggests that, of the three clusters of CK2 phosphorylation sites, only S518/T519/T523 is required for the rapid repair of oxidative SSBs. Although APTX also binds this region of XRCC1, we considered it more likely that the reduced SSBR rate in EM9-XH<sup>S518A/T519A/T523A</sup> cells resulted from the loss of PNK interaction. This is because, in our hands, the loss of APTX activity by itself does not affect global rates of SSBR following oxidative stress (17, 43), whereas the loss of PNK has been reported to slow the rate of the repair of oxidative SSBs (41). To examine directly whether the mutation of S518/T519/T523 slowed chromosomal SSBR rates by disrupting the XRCC1-PNK interaction, we examined whether normal rates of SSBR could be restored in EM9-XH<sup>S518A/T519A/T523A</sup> cells by PNK overexpression (Fig. 5B). Indeed, the rate of chromo-

somal SSBR observed in EM9-XH<sup>S518A/T519A/T523A</sup> cells was restored to normal by the transient overexpression of wild-type recombinant human PNK protein (Fig. 5C). In contrast, PNK<sup>D171N</sup>, harboring a mutated DNA 3'-phosphatase domain, was unable to complement the defect in SSBR in EM9-XH<sup>S518A/T519A/T523A</sup> cells. These data demonstrate, for the first time, that DNA 3'-phosphatase activity is critical for rapid global rates of chromosomal SSBR following oxidative stress, and they suggest that the XRCC1-PNK interaction ensures that this activity does not become rate limiting in vivo.

## DISCUSSION

XRCC1 is a critical component of the SSBR machinery and functions as a molecular scaffold that interacts with multiple enzymatic components of the repair process (9). While numerous XRCC1 partners and their respective binding sites have been identified, it is unclear which of these interactions is important for rapid SSBR rates following oxidative stress in vivo. An understanding of the protein-protein interactions mediated by XRCC1 and their importance during oxidative stress is likely to affect our understanding of human disease, because oxidative stress is a major source of SSBs. Indeed, mutations in components of the SSBR machinery have been identified in



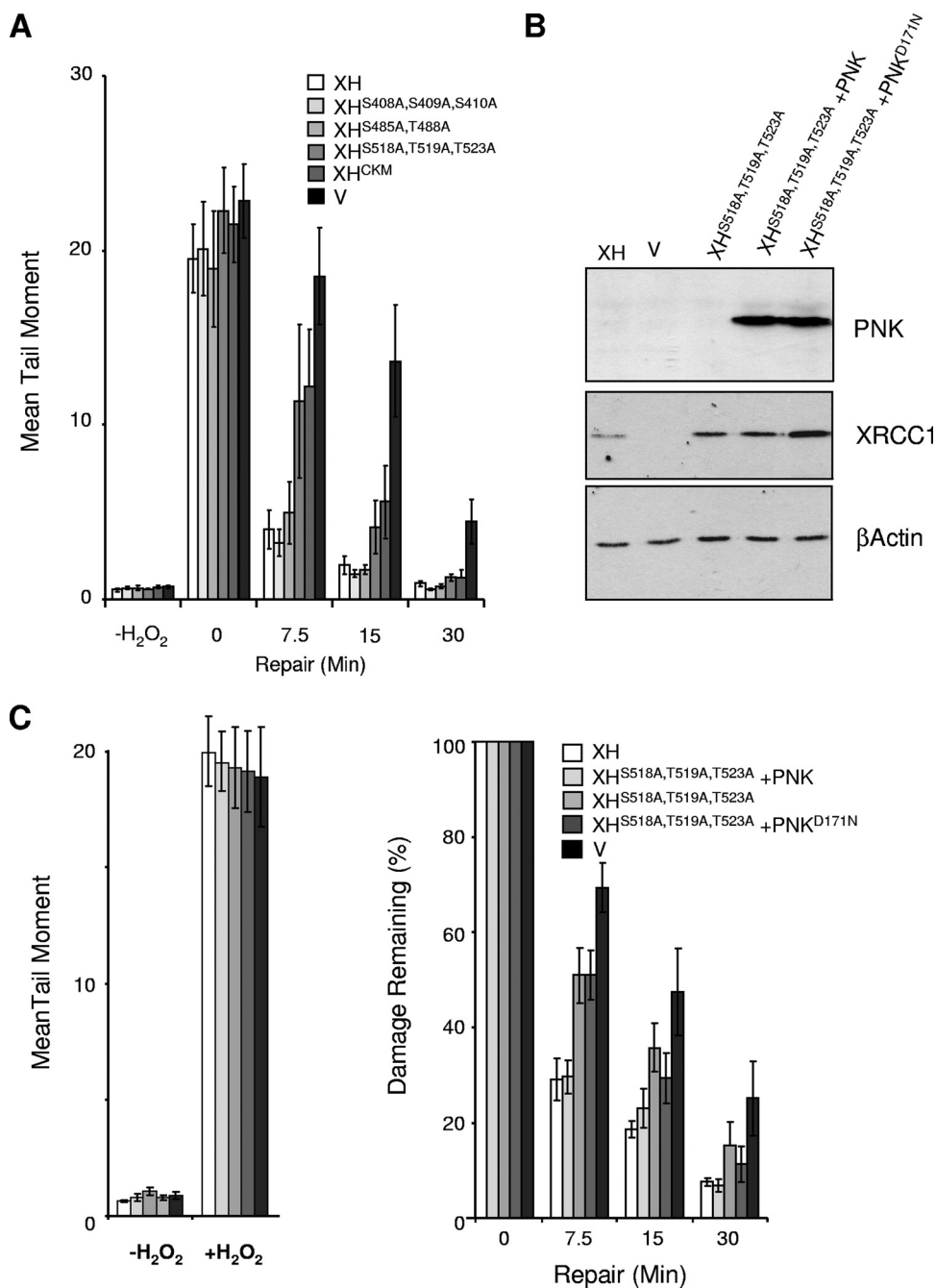


FIG. 5. PNK overexpression overrides the requirement for S518/T519/T523 for rapid global rates of chromosomal SSBR following oxidative stress. (A) DNA strand break repair rates following H<sub>2</sub>O<sub>2</sub> treatment as measured by alkaline comet assays. The indicated EM9 CHO cell lines were treated with 150 μM H<sub>2</sub>O<sub>2</sub> for 20 min on ice in PBS and then incubated for the indicated repair periods in drug-free media. DNA strand breaks were quantified by alkaline comet assays as described in the legend to Fig. 1. Mean tail moments were quantified from ~100 cells/sample/experiment and are the averages (± standard errors of the means) of at least three independent experiments. V, EM9 cells harboring empty vector. (B) Total cell extracts from 3 × 10<sup>4</sup> EM9-XH (XH) or EM9-V (v) cells or EM9-XH<sup>S518A,T519A,T523A</sup> cells transiently transfected with empty vector or with the indicated PNK expression construct were fractionated by SDS-PAGE, transferred to nitrocellulose, and immunostained with anti-PNK polyclonal, anti-XRCC1 monoclonal, or anti-β-actin monoclonal antibody. (C) DNA strand break repair rates following H<sub>2</sub>O<sub>2</sub> treatment as measured by alkaline comet assays. The CHO cells described for panel B were mock treated (-H<sub>2</sub>O<sub>2</sub>) or treated with 150 μM H<sub>2</sub>O<sub>2</sub> (+H<sub>2</sub>O<sub>2</sub>) for 20 min on ice (left) and then incubated for the indicated repair periods in drug-free medium (right). DNA strand breaks were quantified by alkaline comet assays as described above. The data are plotted as the mean fraction of DNA strand breaks remaining and are the averages of at least three independent experiments (± standard errors of the means).

two hereditary neurodegenerative diseases (recently reviewed in references 9 and 42).  
Notably, the XRCC1 mutation F67A failed to prevent XRCC1 from restoring cellular resistance and normal SSBR rates in proliferating EM9 cells following H<sub>2</sub>O<sub>2</sub> treatment or ionizing radiation, despite reducing the interaction with Pol β more than 26-fold. It is unlikely that this reflects residual levels of interaction, because this mutation results in cellular hyper-

sensitivity and reduced SSBR rates following treatment with MMS, which is consistent with the established requirement for this interaction during BER at sites of DNA alkylation (15, 38, 56). Also, His-XRCC1<sup>161-533</sup> protein fully restored rapid global SSBR rates in EM9 cells following H<sub>2</sub>O<sub>2</sub> despite the complete absence of the Pol  $\beta$  binding N-terminal domain in this protein. We conclude from these data that Pol  $\beta$  can function independently of interaction with XRCC1 following oxidative DNA damage or, more likely, that another DNA polymerase can substitute for Pol  $\beta$ . For example, Pol  $\lambda$ , Pol  $\iota$ , and Pol Q also are implicated in the cellular response to H<sub>2</sub>O<sub>2</sub>, and Pol Q has been reported to physically associate with XRCC1 (5, 39, 47, 51, 57). Alternatively, the long-patch repair DNA polymerases Pol  $\delta$  and/or Pol  $\epsilon$  might substitute for Pol  $\beta$ . Consistent with this idea, while early-passage and proliferating populations of Pol  $\beta^{-/-}$  murine embryonic fibroblasts exhibit normal sensitivity and DNA strand break repair rates following oxidative stress, increased sensitivity and decreased repair rates are observed in late-passage or G<sub>1</sub>-enriched Pol  $\beta^{-/-}$  murine embryonic fibroblasts, conditions under which long-patch repair machinery may be downregulated (21, 52, 53). It remains to be determined whether a similar cell cycle stage-specific requirement exists for the interaction of Pol  $\beta$  with XRCC1.

Surprisingly, the deletion of the entire XRCC1 C-terminal BRCT domain (BRCT II) that binds Lig3 $\alpha$  also failed to significantly affect global SSBR rates in asynchronous CHO cells following oxidative stress. Once again, it is unlikely that this reflects residual interaction between XRCC1 and Lig3 $\alpha$ . This is because interaction with XRCC1 is required to stabilize the DNA ligase *in vivo*, yet the cellular level of Lig3 $\alpha$  is no higher in EM9-XH<sup>8529-633</sup> cells expressing XRCC1 that lack the BRCT II domain than in EM9 cells that lack XRCC1 altogether (36). Consequently, either Lig3 $\alpha$  can function independently of XRCC1 following oxidative stress or, more likely, another DNA ligase can substitute for Lig3 $\alpha$  in proliferating CHO cells. One possibility is that Lig1 can fulfill this role, because this DNA ligase is associated with the DNA replication machinery and can conduct BER *in vitro* (29, 35, 40). It will be of interest to examine whether the requirement for XRCC1-Lig3 $\alpha$  interaction during SSBR following oxidative stress exhibits cell cycle specificity, as has been reported for this interaction following ethyl methanesulfonate-induced DNA alkylation (36, 48).

The phosphorylation of XRCC1 by CK2 within a 100-amino-acid region linking the internal (BRCT I) and C-terminal (BRCT II) BRCT domains is required both for PNK interaction and for rapid SSBR rates following H<sub>2</sub>O<sub>2</sub> treatment (30). However, the phosphorylation of this linker region by CK2 also facilitates interactions with the DNA repair proteins APTX and APLF, confusing the issue of which interaction(s) accounts for the importance of this region for rapid SSBR (2, 13, 22, 31). To address this question, we mapped in detail the PNK binding site within the linker region and identified the cluster of CK2 phosphorylation sites in XRCC1 located at S518, T519, and T523 as being critical for this purpose. Intriguingly, S518/T519/T523 is also the APTX binding site, providing an explanation for the observation that XRCC1 associates with these proteins in separate and distinct complexes (31). The mutation of S518/T519/T523 reduced SSBR rates to a level similar to that observed if all eight primary CK2

sites within the linker region were mutated. In contrast, mutation of the remaining CK2 sites by themselves failed to slow SSBR. The CK2 phosphorylation of XRCC1 within the inter-BRCT domain linker region thus accelerates SSBR largely or entirely via S518/T519/T523.

Although S518/T519/T523 mediates interactions with multiple proteins, we considered it likely that it is the PNK interaction that accounts for the importance of these residues for rapid SSBR following H<sub>2</sub>O<sub>2</sub> treatment. This is because a large fraction (>50%) of the breaks induced by oxidative stress are believed to possess 3'-phosphate termini, and thus are putative substrates for the 3'-phosphatase activity of PNK. In addition, so far we have failed to detect an impact of the loss of APTX or APLF alone on rapid global rates of SSBR in proliferating mammalian cells (data not shown). Consistent with this finding, the overexpression of wild-type PNK restored normal rates of SSBR in CHO cells expressing XRCC1-His<sup>S518/T519/T523</sup> following H<sub>2</sub>O<sub>2</sub> treatment, confirming that the requirement for S518/T519/T523 for rapid global SSBR rates reflects the interaction of this region with PNK. Why might the interaction between XRCC1 and PNK be important for rapid chromosomal SSBR rates following oxidative stress? One possibility is that interaction with XRCC1 ensures that PNK activity is not rate limiting during SSBR *in vivo*. This idea is consistent with the observation that XRCC1 stimulates PNK activity *in vitro* at limiting concentrations of the latter enzyme, possibly by dissociating PNK from its DNA product and thereby increasing the enzyme turnover rate (32, 54). However, interaction with XRCC1 also might promote the recruitment or accumulation of PNK at chromosomal SSBs, because the appearance of this protein in subnuclear foci is reduced in EM9-XH<sup>CKM</sup> cells following oxidative stress (30).

Human PNK possesses both 5'-DNA kinase and 3'-DNA phosphatase activities (23, 25). Whereas 5'-hydroxyl termini likely are present at only a minor fraction of DNA strand breaks following oxidative DNA damage, 3'-phosphate termini may be present at 70% or more of all such breaks (20). Consistent with this, the overexpression of PNK harboring a mutated 3'-phosphatase domain failed to complement the SSBR defect in EM9-XH<sup>S518A/T519A/T523A</sup> cells. Although the mutation of D171 does not directly affect the DNA kinase activity of PNK, it is possible that the repair of 5'-hydroxyl termini also is inhibited by the mutation of the 3'-phosphatase domain. This is because the persistence of a 3'-phosphate terminus may inhibit the phosphorylation of an adjacent 5'-hydroxyl terminus (16). Nevertheless, the inability of PNK<sup>D171N</sup> to restore rapid SSBR rates demonstrates directly that 3'-DNA phosphatase activity is crucial for rapid rates of SSBR following oxidative stress. Interestingly, the overexpression of AP endonuclease-1 (APE1), another protein that can process damaged 3' termini, also increases chromosomal SSBR rates in XRCC1 mutant cells following oxidative stress, albeit to less than half the rate present in wild-type cells (45). However, the 3'-phosphatase activity of human APE1 is two to three orders of magnitude less than that of PNK and is unlikely to be a physiologically relevant source of 3'-DNA phosphatase activity *in vivo* (55). Indeed, the impact of mutating XRCC1 at S518A/T519A/T523A on both PNK binding and chromosomal SSBR rates strongly suggests that PNK, in conjunction with XRCC1, is the major source of this activity *in vivo*.

In summary, we demonstrate here for the first time that there is a critical requirement for DNA 3'-phosphatase activity during the repair of chromosomal SSBs following oxidative stress, and we suggest that the XRCC1 interaction with PNK ensures that this activity is not rate limiting in vivo.

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